

Extraction and Library Construction Protocols

The epididymis was dissected free of connective tissue, a small cut made to the cauda and tissue placed in 5 ml of 1x PBS solution for up to 24 hours at 4°C. The epididymal tissue was minced and the released sperm was centrifuged at 6,000 x g, then the supernatant removed, and the pellet resuspended in NIM buffer, to be stored at -80°C until further use. One hundred µl of sperm suspension was sonicated, spun down at 6,000 g, the pellet washed with 1x PBS once, and then combined with 820 µL DNA extraction buffer and 80 µl 0.1M DTT. The sample was incubated at 65°C for 15 minutes. Following this incubation 80 µl proteinase K (20 mg/ml) was added and the sample incubated at 55°C for at least 2 hours under constant rotation. Then 300 µl of protein precipitation solution (Promega, A7953) was added, the sample mixed thoroughly and incubated for 15 min on ice. The sample was centrifuged at 14,000 rpm for 30 minutes at 4°C. One ml of the supernatant was transferred to a 2 ml tube and 2 µl of glycoblu and 1 ml of cold 100 % isopropanol were added. The sample was mixed well by inverting the tube several times then left in -20°C freezer for at least one hour. After precipitation the sample was centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was taken off and discarded without disturbing the (blue) pellet. The pellet was washed with 70% cold ethanol by adding 500 µl of 70% ethanol to the pellet and returning the tube to the freezer for 20 minutes. After the incubation the tube was centrifuged for 10 min at 4°C at 14,000 rpm and the supernatant discarded. The tube was spun again briefly to collect residual ethanol to bottom of tube and then as much liquid as possible was removed with gel loading tip. Pellet was air-dried at RT until it looked dry (about 5 minutes). Pellet was then resuspended in 100 µl of nuclease free water. Methylated DNA Immunoprecipitation (MeDIP) with genomic DNA was performed as follows: rat sperm DNA pools were generated using the appropriate amount of genomic DNA from each individual for 3 pools each of control and vinclozolin lineage animals. Genomic DNA was sonicated using the Covaris M220 the following way: the pooled genomic DNA was diluted to 130 µl with TE buffer into the appropriate Covaris tube. Covaris was set to 300 bp program and the program was run for each tube in the experiment. 10 µl of each sonicated DNA was run on 1.5% agarose gel to verify fragment size. The sonicated DNA was transferred from the Covaris tube to a 1.7 ml microfuge tube and the volume measured. The sonicated DNA was then diluted with TE buffer (10mM Tris HCl, pH7.5; 1mM EDTA) to 400 µl, heat-denatured for 10 min at 95°C, then immediately cooled on ice for 10 min. Then 100 µl of 5X IP buffer and 5 µg of antibody (monoclonal mouse anti 5-methyl cytidine; Diagenode #C15200006) were added to the denatured sonicated DNA. The DNA-antibody mixture was incubated overnight on a rotator at 4°C.

The following day magnetic beads (Dynabeads M-280 Sheep anti-Mouse IgG; 11201D) were pre-washed as follows: The beads were resuspended in the vial, then the appropriate volume (50 µl per sample) was transferred to a microfuge tube. The same volume of Washing Buffer (at least 1 mL 1XPBS with 0.1% BSA and 2mM EDTA) was added and the bead sample was resuspended. Tube was then placed into a magnetic rack for 1-2 minutes and the supernatant discarded. The tube was removed from the magnetic rack and the beads washed once. The washed beads were resuspended in the same volume of 1xIP buffer (50 mM sodium phosphate pH7.0, 700 mM NaCl, 0.25% TritonX-100) as the initial volume of beads. 50µl of beads were added to the 500µl of DNA-antibody mixture from the overnight incubation, then incubated for 2h on a rotator at 4°C.

After the incubation the bead-antibody-DNA complex was washed three times with 1X IP buffer as follows: The tube was placed into magnetic rack for 1-2 minutes and the supernatant discarded, then washed with 1xIP buffer 3 times. The washed bead-DNA solution is then resuspended in 250 µl digestion buffer with 3.5 µl Proteinase K (20mg/ml). The sample was then incubated for 2-3 hours on a rotator at 55° C and then 250 µl of buffered Phenol-Chloroform-Isoamylalcohol solution was added to the supe and the tube vortexed for 30 sec then centrifuged at 14,000rpm for 5 min at room temperature. The aqueous supernatant was carefully removed and transferred to a fresh microfuge tube. Then 250 µl chloroform were added to the supernatant from the previous step, vortexed for 30 sec and centrifuged at 14,000rpm for 5 min at room temperature. The aqueous supernatant was removed and transferred to a fresh microfuge tube. To the supernatant 2µl of glycoblu (20mg/ml), 20µl of 5M NaCl and 500µl ethanol were

added and mixed well, then precipitated in -20°C freezer for 1 hour to overnight.

The precipitate was centrifuged at 14,000rpm for 20min at 4°C and the supernatant removed, while not disturbing the pellet. The pellet was washed with 500µl cold 70% ethanol in -20°C freezer for 15 min. then centrifuged again at 14,000rpm for 5min at 4°C and the supernatant discarded. The tube was spun again briefly to collect residual ethanol to bottom of tube and as much liquid as possible was removed with gel loading tip. Pellet was air-dried at RT until it looked dry (about 5 minutes) then resuspended in 20µl H₂O or TE. DNA concentration was measured in Qubit (Life Technologies) with ssDNA kit (Molecular Probes Q10212). Histone chromatin immunoprecipitation with genomic DNA was performed as follows: rat sperm pools were generated using a total of 8 million sperm per pool for 3 pools of control and vinclozolin lineage animals. The control pools contained equal amounts of sperm for each of 3-4 individuals for a total of n=11 rats and the vinclozolin pools contained equal amounts of sperm for each of 3 individuals for a total of n=9 rats per exposure group. To remove any somatic cell contamination sperm samples from each animal were sonicated 10 seconds using a Sonic Dismembrator Model 300 (Thermo Scientific Fisher, USA) then centrifuged 4,000xg for 5 min at 4°C. The supernatant was discarded and the pellet resuspended and counted individually on a Neubauer counting chamber (Propper Manufacturing Co., Inc., New York, USA) prior to pooling. The sperm pools were reconstituted up to 1 ml with 1X PBS. To reduce disulfide bonds, 50 µl of 1M DTT was added to each pool and the pools were then incubated for 2 hours at room temperature under constant rotation. To quench any residual DTT in the reaction, 120 µl of fresh 1M NEM (N-Ethylmaleimide, Thermo Scientific, Rockford, USA) was then added and the samples incubated for 30 min at room temperature under constant rotation. The sperm cells were pelleted at 2,000g for 5 min at room temperature and the supernatant discarded. Pellets were resuspended in 1X PBS and then spun again at 2,000g for 5 min at room temperature. The supernatant was discarded. The sperm cells were then resuspended in "buffer 1" (final concentration: 15 mM Tris-HCl (pH 7.5), 60 mM KCl, 5 mM MgCl₂, 0.1 mM EGTA; all filtered through a 0.22 µm filter and stored at room temperature) in a ratio of 2 million sperm cells per 50 µl. "Complete buffer" was "buffer 1" supplemented with 0.5% tergitol (vol/vol) and 1% DOC (wt/vol) (sodium deoxycholate, Sigma Aldrich 30970). 50 µl of this supplemented buffer was added to each aliquot. The samples were mixed and incubated for 20 min on ice. Sperm cell DNA was divided into aliquots of 4 µg of DNA. These aliquots were sonicated using the Covaris M220 the following way: 4 µg of genomic DNA was resuspended in 130 µl of complete buffer supplemented with tergitol 0.5% and DOC 1%. Covaris was set to a 10 min "Chromatin shearing" program and the program was run for each tube in the experiment.

For each sample 10 µl was run on a 1.5% agarose gel to verify fragment size. Aliquoted samples were pooled back together and centrifuged at 12,500 rpm for 10 min at room temperature. The supernatant was transferred to a fresh microfuge tube. 65 µl of protease inhibitor cocktail (1 tablet dissolved in 500 µl, 20x concentrated) (Roche, cat. no. 11 873 580 001) were added to each sample as well as 3 µl of antibody (monoclonal rabbit anti-histone H3, Millipore 05-928). The DNA-antibody mixture was incubated overnight on a rotator at 4°C. The following day, magnetic beads (ChIP-Grade protein G magnetic beads, Cell Signaling 9006) were pre-washed as follows: the beads were resuspended in the vial, then 30 µl per sample was transferred to a microfuge tube. The same volume of Washing Buffer (at least 1 ml) was added and the bead sample was resuspended. Tube was then placed into a magnetic rack for 1-2 minutes and the supernatant discarded. The tube was removed from the magnetic rack and the beads washed once. The washed beads were resuspended in the same volume of 1X IP buffer as the initial volume of beads. 30 µl of beads were added to each DNA-antibody mixture from the overnight incubation, then incubated for 2h on a rotator at 4°C. After the incubation, the bead-antibody-DNA complex was washed three times with 1X IP buffer as follows: the tube was placed into a magnetic rack for 1-2 minutes and the supernatant discarded, then washed with 1X IP buffer 3 times. The washed bead-antibody-DNA solution was then resuspended in 300 µl of digestion buffer and 3 µl proteinase K (20 mg/ml). The sample was incubated for 3h on a rotator at 56°C. After incubation the samples were extracted with Phenol-Chloroform-Isoamylalcohol and precipitated with 2 µl of Glycoblue (20 mg/ml), a one-tenth volume of 3M sodium acetate and two volumes of ethanol overnight at -20°C.

The precipitate was centrifuged at 14,000 rpm for 30 min at 4°C and the supernatant removed, while not disturbing the pellet. The pellet was washed with 500 µl cold 70% ethanol, then centrifuged again at 14,000 rpm for 10 min at 4°C and the supernatant discarded. The tube was spun briefly to collect residual ethanol to bottom of tube and as much liquid as possible was removed with a gel loading tip. Pellet was air-dried at RT until it looked dry (about 5 min) then resuspended in 20 µl H₂O. DNA concentration was measured in the Qubit (Life Technologies) with the BR dsDNA kit (Molecular Probes Q32853)

